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Antibody scFv Fragments Without Disulfide Bonds Made by Molecular Evolution

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We generated stable and functional cysteine-free antibody single-chain fragments (scFv) lacking the conserved disulfide bonds in both V_H and V_L. This was achieved by molecular evolution, starting from the scFv fragment of the levan binding antibody ABPC48, which is naturally missing one of the conserved cysteine residues, by using DNA shuffling and phage display. Several of the selected sequences were expressed and the resulting scFv proteins characterized by equilibrium urea denaturation. Three of the characterized proteins exhibit thermodynamic stability similar to the wild-type protein, and these cysteine-free mutant proteins can now be expressed in functional form in the *Escherichia coli* cytoplasm. We believe that such molecules are of great utility for use as intrabodies, can be produced by simpler expression strategies and may give further insight into the folding and stability of the immunoglobulin fold.

Keywords: disulfide bond; antibody engineering; scFv fragment; intrabodies; cytoplasmic expression

Introduction

The disulfide bond significantly contributes to the stability of antibody domains. Most antibodies will not tolerate its loss and will react to its removal with a dramatic loss of free energy of folding. However, there are a number of interesting applications for antibody fragments in environments that are not compatible with efficient disulfide bridge formation, such as the expression of functional antibody fragments in the reducing environment of the cytoplasm. Such "intrabodies" might be used to bind and inactivate cellular components (Richardson & Marasco, 1995; Biocca & Cattaneo, 1995). In the long run, such molecules may also be useful for a direct coupling of the antibody-antigen interaction to transcription, thereby linking affinity to bacterial growth (*via* an antibiotic resistance gene) or automated detection (*via* a gene for a colorigenic enzyme). In the case of intrabodies derived from catalytic antibodies, metabolic selection schemes may be used to enhance their activities or even alter metabolic pathways. Since normal antibody fragments do not form disulfide bonds in the cytoplasm (Biocca *et al.*, 1995), and usually are unable to achieve a stable native fold in the absence of the disulfide bonds, the reported activities of cytoplasmic intrabodies may be due to low-stability species or even only partially folded molecules. Consequently, there is great interest in engineering antibody fragments that will fold and are stable under reducing conditions, and that could serve as framework to which other specificities could be grafted.

The disulfide bond connecting strand b (position L23 or H22, numbered according to Kabat *et al.*, 1991) and strand f (L88 or H92) of the antibody variable domains links the two beta sheets of the immunoglobulin domain. It is widely maintained within the immunoglobulin superfamily and almost perfectly conserved in antibody variable domains. The stabilizing effect of the disulfide bonds has been tested experimentally (Goto & Hamaguchi, 1979), and for the antibody McPC603 no functional antibody fragment could be obtained if any of these cysteine residues had been replaced (Glockshuber *et al.*, 1992). The few antibodies that are functional despite having lost one of the conserved cysteine residues through somatic mutation (Rudikoff & Pumphrey, 1986) appear to have

Abbreviations used: BBS, borate-buffered saline; BMS, bis-2-mercaptoethylsulfone; CDR, complementarity determining region; dNTP, deoxynucleotide triphosphate; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; Fv, variable fragment of an antibody; GdnHCl, guanidinium hydrochloride; Ig, immunoglobulin; IPTG, isopropyl-β-D-thiogalactopyranoside; PVDF, polyvinyldifluoride; scFv, single-chain variable fragment of an antibody; TBS, Tris buffered saline; V_H, variable domain of antibody heavy chain; V_L, variable domain of antibody light chain.

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above average thermodynamic stability after the missing disulfide bond has been restored (Proba *et al.*, 1997). Globally stabilizing mutations have been shown to permit removal of the disulfide bond from the V_L domain REI (Frisch *et al.*, 1994, 1996).

We have undertaken a molecular evolution study in which we selected for the best replacements for the cysteine residues as well as for mutations that may compensate for the loss in stability upon removal of both disulfide bonds in a scFv fragment. We generated and characterized such fragments, which fold correctly to stable structures and are fully functional in the absence of any cysteine residues.

Results and Discussion

Shuffling and selection

As the starting point for the generation of functional cysteine-free scFvs, the scFv fragment of the antibody ABPC48 was chosen. This antibody naturally carries tyrosine in place of Cys-H92 (Rudikoff & Pumphrey, 1986) and yields functional, monomeric scFv fragments lacking the V_H disulfide bond (Proba *et al.*, 1997). Based on this scFv (referred to as A48wt) in the V_H-$(Gly_4Ser)_6$-V_L format, two small libraries were constructed. In one, the unpaired Cys-H22 was replaced by Ser, Ala, Val or Leu, all in the presence of Tyr-H92, while the disulfide-containing V_L domain was left unchanged. In the other library, Tyr-H92 was replaced by Cys to restore the V_H disulfide bond, while the two cysteine residues of the V_L domain were replaced by Ala, Val, Leu, Ile or Phe. The two libraries were inserted into the phagemid pAK100 (Krebber *et al.*, 1997) and functional variants selected from either library through three rounds of phage panning. All four initial substitutions of Cys-H22 survived panning, with H22-Ala and H22-Val found most frequently in the presence of Tyr-H92. In the case of the V_L domain, of the 25 initial combinations only the substitutions L23-Ala/L88-Ala and L23-Val/L88-Ala were found after panning (Figure 1a).

In a second step, the disulfide-free V_H domains (H22-Ala/H92-Tyr or H22-Val/H92-Tyr) were randomly combined with the disulfide-free V_L domains (L23-Ala/L88-Ala or L23-Val/L88-Ala) and submitted to DNA-shuffling and random mutagenesis according to Stemmer (1994). The resulting scFv DNA fragments, assembled and amplified from the shuffling mixture, were ligated and transformed, resulting in about 10^4 clones (library I). Out of 24 clones of this library analyzed before panning, 12 contained correctly assembled scFv sequences. Of these, three coded for the unaltered amino acid sequence, five contained one, and four sequences contained two amino acid substitutions. Due to the highly repetitive nature of the $(Gly_4Ser)_6$-linker, which allows for different ways of fragment assembly in the shuffling procedure, we found a distribution of linker lengths from 10 to 35 amino acid residues. Since short linkers can cause diabody formation in scFvs (Holliger *et al.*, 1993; Desplancq *et al.*, 1994; Whitlow *et al.*, 1994) and avidity effects cause such diabodies to be enriched in phage panning (Schier *et al.*, 1996), we decided to change to a 20-mer non-repetitive linker (Tang *et al.*, 1996).

Using the unselected scFv PCR product from the first shuffling experiment as template, a second shuffling round was performed to introduce the new linker and to introduce additional mutations. Ligation of the scFv PCR product and transformation resulted in library II, containing 1.3×10^4 clones. Libraries I and II were each subjected to three rounds of phage panning. Since the initial disulfide-free scFv fragments were expected to have low thermodynamic stability, phage propagation was carried out at 24°C. Ten clones from each library were analyzed. Nine of ten clones from library I and two of ten from library II contained a disulfide-bond in the V_H domain, probably due to contamination of the libraries with the original, disulfide-restored V_H domain. Three different disulfide-free sequences were found that all contained Ala-H22, Val-L23 and Ala-L88 at the former cysteine positions, H92 being Tyr as in the wild-type, plus a number of additional mutations (Figure 1b). Clone A48I-1, derived from library I, contained a Gly/Ser linker shortened to 24 residues, while clones A48II-1 and A48II-2, derived from library II, contained the non-repetitive linker in unaltered form.

A48II-1 and A48II-2 were mixed with A48I-1 in different proportions, and oligonucleotides coding for the non-repetitive 20-mer linker and for Arg and Glu at position H22 were added, since modelling suggested that H22 is solvent-exposed (Proba *et al.*, 1997). These mixtures were used as templates for a third round of shuffling. The resulting libraries (IIIa, IIIb and IIIc), which were 5×10^4 to 7×10^4 in size, were combined, and five rounds of phage panning were performed, with increased stringency of selection in the fourth and fifth round (increasing the temperature during phage propagation to 28°C and 30°C, and addition of 0.5 M GdnHCl during binding incubation in the panning tube). Amongst 21 clones sequenced after the third round of panning, two clones (A48III-1 and A48III-2) were enriched (Figure 1c). The same two sequences were dominant after the fourth round. A V_L disulfide bond revertant appeared in this round and became dominant after the fifth round.

Characterization of dominant clones

Clones isolated from libraries I and II (A48I-1 and A48II-1) showed low but significant specific binding in phage-ELISA, while clones from library III (A48III-1, -2 and -3) showed dramatically increased phage-ELISA signals, which are in the range of the A48wt signal (Figure 2). For further

characterization, scFvs A48wt, A48I-1, A48II-1, A48II-2, A48III-1, A48III-2 and A48III-3 were produced as inclusion bodies in *Escherichia coli*, refolded and purified by antigen affinity chromatography as described (Proba *et al.*, 1997). In addition, A48wt and A48I-1, which originally con-

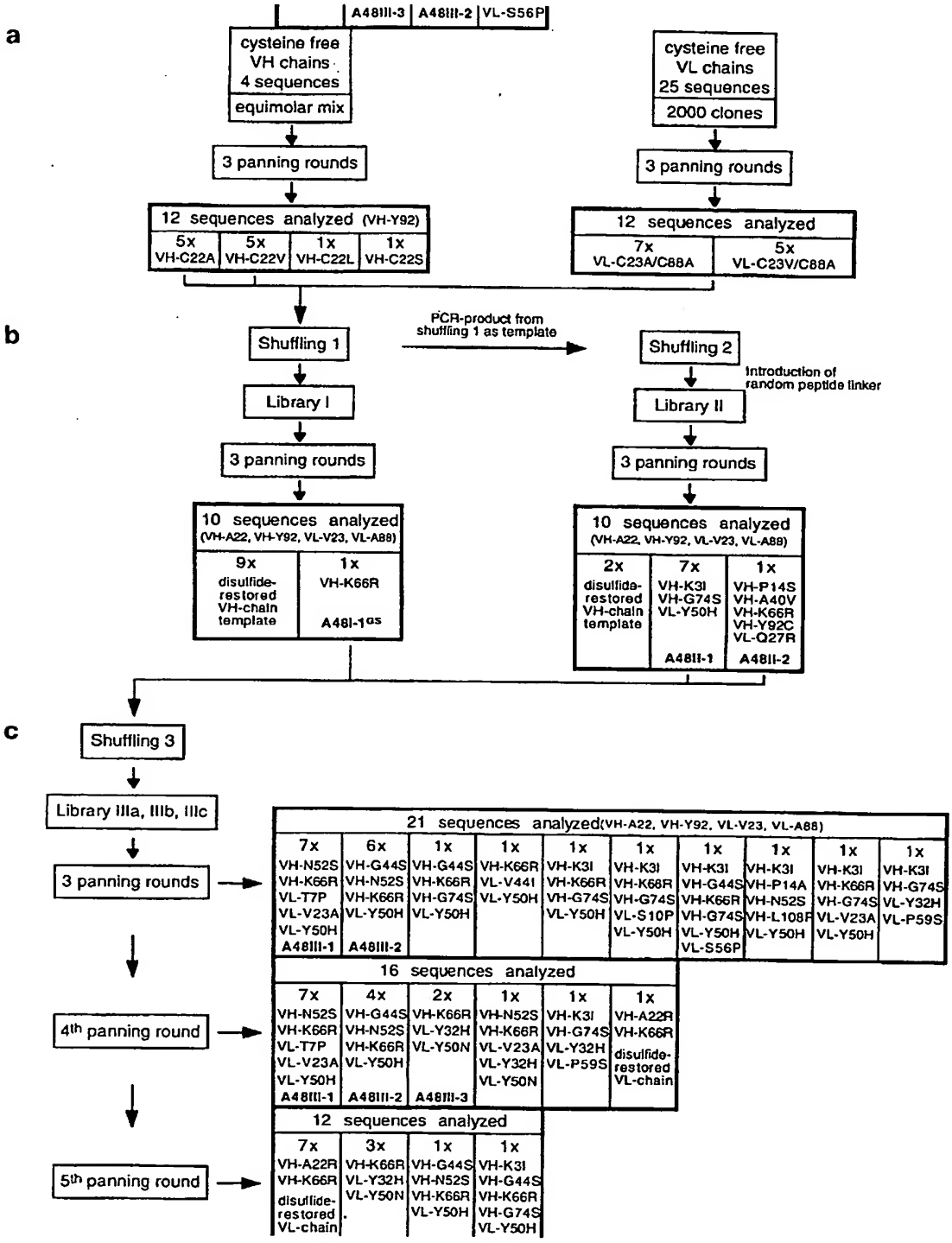


Figure 1. A representation of DNA shuffling and panning experiments with mutants selected. a, Single-domain libraries; b, first and second combination and shuffling; c, third round of shuffling.

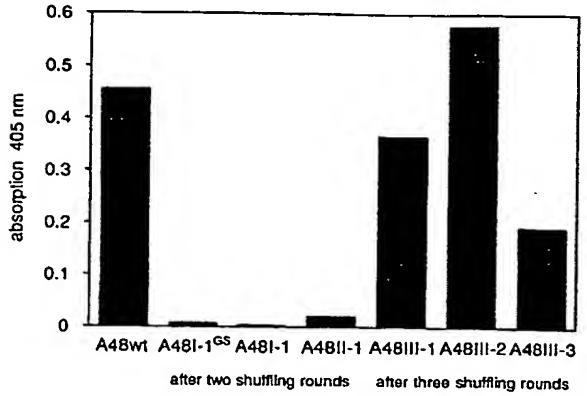


Figure 2. ELISA signals of phages displaying the scFvs A48wt, A48I-1, A48I-1^{GS} (containing $(Gly_4Ser)_6$-linker), A48II-1, A48III-1, A48III-2 and A48III-3. All scFvs except A48I-1^{GS} contain the non-repetitive 20-mer linker. Titers of the phages under investigation were identical within a factor of 2 of each other. ELISA signals are normalized to a constant phase number. Specificity of binding was confirmed by addition of antigen (0.025% bacterial levan), which suppressed the signals to zero in all cases.

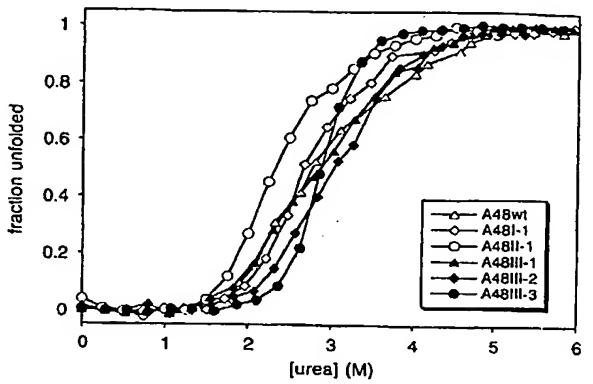


Figure 3. Urea denaturation curves of wild-type and mutant scFv proteins obtained after DNA shuffling and panning. All scFvs contain the non-repetitive 20-mer linker.

tained the Gly/Ser linker, were also produced with the non-repetitive 20-mer linker to assess the influence of the different linkers on stability. Typical yields were 15 to 25 mg of functional protein per liter of *E. coli* culture, with the exception of A48II-2, which, most likely due to the presence of the unpaired Cys-H92, yielded only 0.1 mg/l. This mutant was not further examined. A disulfide-free construct without any further mutations (L23-Val/L88-Ala, H22-Ala/H92-Tyr) could not be refolded, which directly demonstrates the importance of the additional selected mutations.

The thermodynamic stability of the proteins was determined by urea equilibrium denaturation. The denaturation curve of A48wt carrying the $(Gly_4Ser)_6$ linker and that of A48wt carrying the non-repetitive 20-mer linker were virtually identical (data not shown). A48II-1 shows a marked decrease in stability compared to A48wt (Figure 3), while the stabilities of A48I-1, A48III-1 and A48III-2 were close to that of A48wt, indicating the presence of stabilizing mutations that compensate for the loss incurred through the removal of the V_L disulfide bond. A48III-3 shows a similar denaturation midpoint to A48wt, but the curve is steeper. While this may indicate an increased cooperativity of the unfolding transition, it may also be a consequence of the observed curves being a sum of two unfolding curves, one for each domain, where a small shift in one curve will create the appearance of a different steepness. The free energy of folding could not be calculated from the presented urea denaturation curves, because unfolding of the proteins obviously does not follow a two-state model of unfolding.

Extracts of soluble cytoplasmic protein analyzed by Western blot showed similar amounts of total soluble A48wt scFv and A48III-1 scFv (Figure 4a). However, in an antigen-binding assay, the ELISA signals obtained from A48III-1 cytoplasmic extracts were significantly higher than those obtained with A48wt extracts (Figure 4b), demonstrating that A48III-1 protein is functional in the cytoplasm. It is very unlikely that differences in antigen affinity contribute to differences in the ELISA-signals in Figure 4b, because the A48wt scFv and A48III-1 scFv exhibit similar signals in phage ELISA (Figure 2). In addition, inhibition ELISA carried out with identical concentrations of the purified proteins show almost identical signal intensities and inhibition behavior (data not shown). In the case of A48wt, a small fraction of the protein was present in oxidized form, dependent on the work-up procedure used, probably due to air oxidation. The intensity of this band (Figure 4a) seems to correspond to the background activity of A48wt (Figure 4b). We thus conclude that more functional protein of the mutant A48III-1 than of the A48wt can be obtained from the reducing cytoplasm.

While the urea denaturation curves show only small further improvement over the single mutation Lys-H66-Arg in clone A48I-1, phage ELISA signals are significantly higher in clones from library III than from libraries I or II (Figure 2). This indicates that further selection is occurring, not only for reasons of stability, but rather for combined properties including stability, folding yield and/or binding affinity. The result of the phage ELISA clearly shows that the signal intensity in phage ELISA by itself is not useful for identifying mutants that are more stable. The signal intensity in phage ELISA depends on a variety of parameters, such as folding efficiency, binding affinity, toxicity for the producing host and stability. The scFv proteins with essentially identical stability (A48wt and A48I-1, Figure 3) can differ signifi-

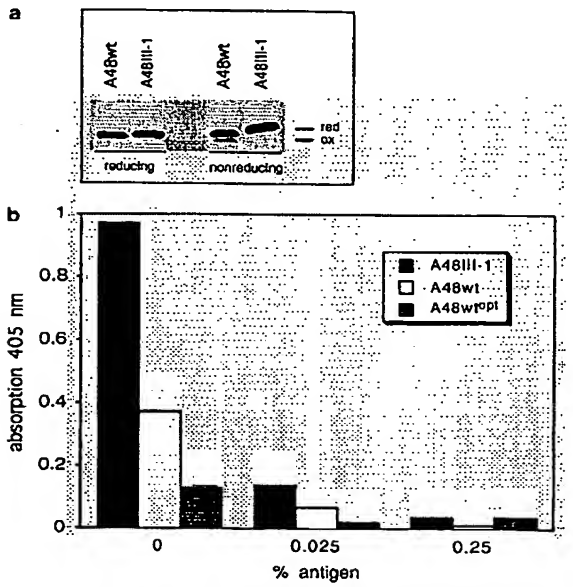


Figure 4. a, Western blot analysis of soluble fractions of *E. coli* cultures expressing A48wt and A48III-1 in the cytoplasm. Samples were prepared as described in Materials and Methods, separated in the presence (reducing) or absence (non-reducing) of β-mercaptoethanol in the SDS-PAGE loading buffer. After blotting, the scFvs were detected with the anti-myc antibody 9E10. b, ELISA of soluble fractions of *E. coli* cultures expressing A48wt and A48III-1 in the cytoplasm, and inhibition by bacterial levan. The amount of soluble scFv in the crude extracts tested was approximately equal, based on Western blot analysis. The intensity of the signals produced by A48wt were strongly dependent on the buffer used for sample preparation. Buffer conditions allowing oxidation after cell disruption and during ELISA (Tris (pH 7.5), no reducing agent) gave rise to a higher signal in the absence of inhibitor (A48wt) than optimized buffer conditions ($A48wt^{opt}$), making air oxidation of free thiol groups more difficult (sodium phosphate (pH 6.0), 5 mM bis-2-mercaptoethylsulfone).

cantly in signal intensity (Figure 2), because phage ELISA samples the overall functionality of scFv displaying phages, rather than single contributions. More detailed experiments with single mutations will be required to disentangle the effects of each substitution to fully understand the biophysical basis of the selection of each mutant.

Structural models: replacement of cysteine residues

Based on the high reactivity of the unpaired cysteine residue in the functional A48wt scFv fragment and on the observation that quantitative modification of the unpaired cysteine with glutathione did not prevent refolding to full activity nor impair the stability of the refolded scFv fragment, an unusual structural alteration of the V_H domain has been proposed earlier (Proba *et al.* 1997). We suggested that, through a local change of the main-chain conformations in strand b, the unpaired Cys-H22 faces outwards rather than being buried in the core of the domain (Figure 5a). This structural alteration is stabilized by hydrogen bonds between the side-chain OH group of Tyr-H92, which in A48 replaces Cys-H92, and main-chain atoms of strands b and e. Indeed, it was observed in the panning of the initial V_H library that Cys-H22 can be replaced by larger residues and even by Arg without any impairment of function (Figure 1c).

In contrast, only substitutions of Cys-L23 and Cys-L88 by Ala or Val can be accommodated in the core of the V_L domain without structural alteration. Although larger residues were also included in the initial V_L library, a clear selection for Ala-L23/Ala-L88 and Val-L23/Ala-L88 occurred. Larger residues incompatible with a conventional packing of the V_L core disappeared within the first three rounds of panning. In the course of further shuffling and panning rounds, Val/Ala (65/81 clones) clearly dominated over Ala/Ala (16/81 clones), which occurred predominantly in conjunction with the mutation of Thr-L7 to Pro (14/81) clones. In a systematic study of substitutions of the disulfide-forming Cys30 and Cys51 in bovine pancreatic trypsin inhibitor, the combination valine/alanine was also found as the least destabilizing replacement (Liu *et al.*, 1997).

Structural basis of selected mutations

A construct containing only the disulfide replacement without further mutations, and thus representing the starting point of the experiment, could not even be refolded in sufficient yield to allow the determination of its stability. In contrast, the selected clones could all be refolded and analyzed. The most frequently observed mutation is Lys-H66 to Arg. It occurred in 48 out of 81 clones sequenced and persisted through all rounds of panning. Urea denaturation curves showed that clone A48I-1, containing only this mutation, is as stable as the disulfide-containing wild-type and markedly more stable than a clone retaining Lys-H66 (A48II-1) but containing other stabilizing mutations.

Residue H66 is part of a highly conserved charge cluster within the V_H domain (Figure 5b). Of all human V_H sequences, 90.4% contain Arg in this position and only 1.3% Lys, while mouse V_H domains contain 47.1% Arg and 51.4% Lys. Residue H66 interacts strongly with Asp-H86 (98.5% of all human and 98.8% of all mouse sequences). In X-ray structures of V_H domains that naturally contain Arg-H66 (38 out of 65 V_H domain structures in the PDB database), this residue forms an ideal interaction with Asp-H86, with the two guanidino NH groups of Arg forming hydrogen bonds to the two carboxylate oxygen atoms of Asp. This salt-bridge is quite buried, increasing its stabilizing effect compared to that of a solvent-exposed ion pair.

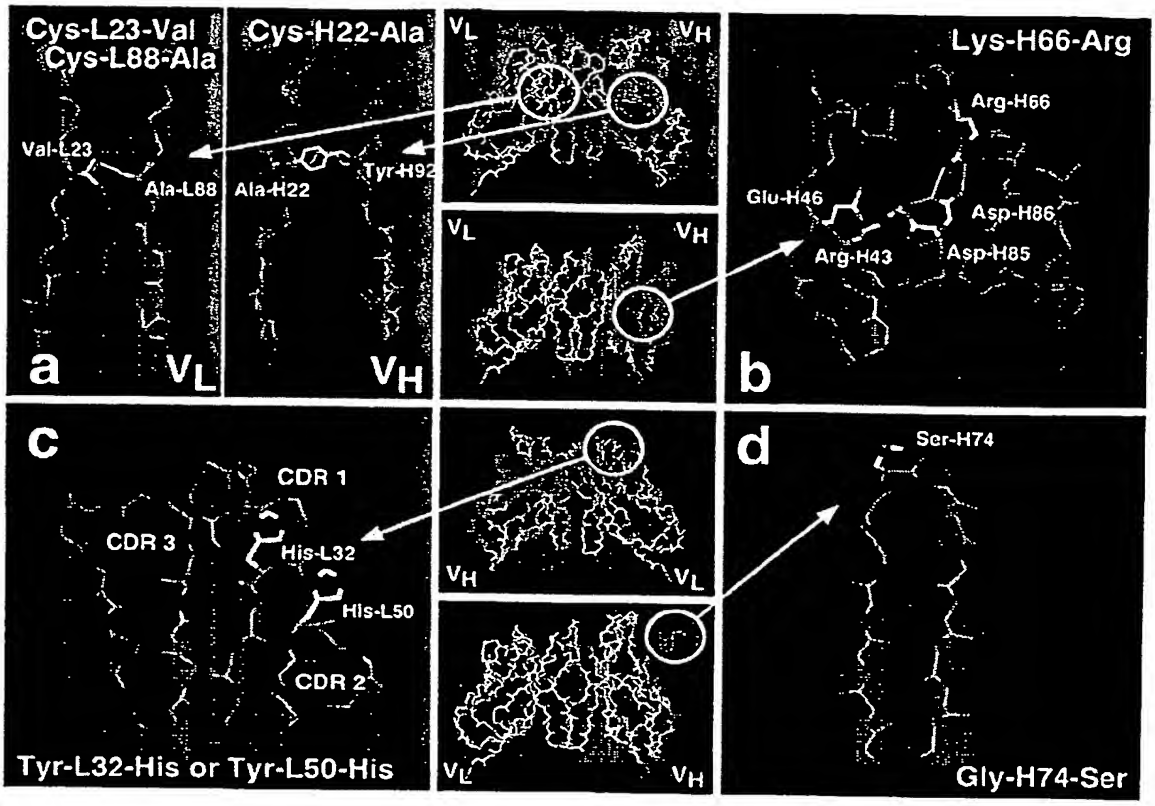


Figure 5. Position of key mutations in the model structure. a, Disulfide-free (thick lines) and disulfide-restored (thin lines) A48: conformation of strands b and f. b, Residues interacting with Lys-H66-Arg. Local side-chain conformations and hydrogen-bonding pattern of the cluster of charged residues interacting with Arg-H66 were modelled according to the PDB file 7fab (Saul & Poljak, 1992); c, Tyr-L32-His or Tyr-L50-His; d, Gly-H74-Ser.

Mutations Tyr-L50 to His (40 clones) and Tyr-L32 to His (seven clones) are located right next to each other in the model structure of ABPC48 (Figure 5c). In six of seven cases, Tyr-L32-His is combined with Tyr-L50-Asn. The position of the mutations in CDR L1 and CDR L2 makes an effect on antigen affinity very likely. This is confirmed by the significantly increased phage ELISA signal of A48III-1, -2 and -3 compared to that of A48I-1. Unfortunately, the heterogeneous oligomeric nature of the antigen precludes the determination of exact binding constants.

Gly-H74-Ser (Figure 5d), present in 17 of 81 clones, is located at the tip of the hairpin turn forming the "outer loop" of the V_H domain. Gly-H74-Ser represents a mutation towards the sequence consensus. Mouse sequences contain 76% Ser, 14% Ala, 7% Pro and less than 0.25% Gly in this position; human V_H domains contain 84% Ser, 10% Ala, 2% Pro and no Gly. This mutation may help to form the beta-turn and may influence folding kinetics as well as stability.

Conclusions

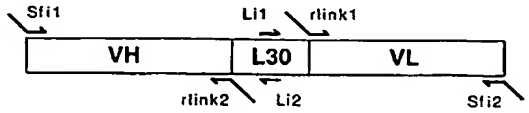
The stability loss caused by the substitution of Cys-L23-Val and Cys-L88-Ala in the A48wt scFv was compensated by the stabilizing effect of the substitution of Lys-H66 by Arg, selected within the first few rounds of mutagenesis. Further rounds lead to improvements of display phage functionality, which was not reflected in greatly improved scFv stability, but probably primarily affected binding affinity and maybe folding kinetics and yield, although to link these effects to individual mutations will require further analysis of single mutations. The scFv fragments generated in this work are at least as functional as the original sequence of ABPC48, without requiring any disulfide bond formation. It remains to be investigated if further improvements can be achieved by increasing selection stringency, and whether the frameworks selected by this strategy can indeed be used to generate more active intrabodies.

Materials and Methods

Phagemid constructs

The scFv genes of A48wt and A48-Cys in the V_H-$(Gly_4Ser)_6$-V_L format were amplified from the periplasmic expression plasmids (Proba *et al.*, 1997), using the primers Sfi1 and Sfi2 (Figure 6). To perform the PCR reactions, Vent DNA polymerase (NEB) was used. The PCR products were inserted into the *Sfi*I restriction site

a



b

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Sfi1      5'-CTACTCGCGGCCCAGCCGGCCATGGCCGACTACAAAGACGAAG-3'
Sfi2      5'-CCGAATTCGGCCCCGAGGCCCGCTTAAGTTCCAGTTTAGTACC-3'
Li1       5'-TGGTGGGAGCTCTGGTGG-3'
Li2       5'-ACCACCAGAGCTCCCACC-3'
rlink1    5'-CACCAGGTTCCACTCCGCAGGCGGTCCGACTGCTAACTCTGGAGA
            TATCCAGATGACCCAGACC-3'
rlink2    5'-CCTGCGGATGGAACCTCGTGGGAGTTGTGAGACGGGGTAGAGCT
            CACGGTAACCAGGGTACCCTG-3'
A-H22-E   5'-CTGTCTCAACCTGCCTCAGGTTTCG-3'
A-H22-R   5'-CTGTCTCGTGCTGCCTCAGGTTTCG-3'
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c

ThrProSerHisAsnSerHisGlnValProSerAlaGlyGlyProThrAlaAsnSerGly

Figure 6. a, A representation of the primers used for DNA shuffling experiments. b, Primer sequences. c, Amino acid sequence of the non-repetitive 20-mer linker.

of the vector pAK100 (Krebber *et al.*, 1997) using standard cloning methods.

DNA shuffling

V_H and V_L genes were amplified from appropriate templates (see Results and Discussion) using the primer pairs Sfi1/Li2 (V_H) and Li1/Sfi2 (V_L) or, for introduction of the 20-mer non-repeptitive linker, primer pairs Sfi1/rlink2 and rlink1/Sfi2. To amplify complete scFv genes, the primer pair Sfi1/Sfi2 was used (Figure 6). PCR reactions were performed as above, except elongation times at 72°C were adapted to the expected product length. The resulting PCR products were purified from 1 to 1.3% agarose/TAE gels using the Qiaex I gel extraction system (Qiagen) and usually eluted in 100 µl of 10 mM Tris-HCl (pH 8).

The DNA shuffling procedure was applied to the purified PCR products according to Stemmer (1994) with the following modifications. (i) After DNase digestion, DNA fragments in the 100 to 250 bp range were purified from 1.3% agarose/TAE gels using the Qiaex I gel extraction system. (ii) The yield of purified DNA fragments resulting from DNase-digestions was not estimated prior to shuffling PCR. Therefore, shuffling PCR was carried out in triplicate, using different amounts of DNase digested fragments (36, 12 and 4 µl out of 100 µl) as template. (iii) The shuffling PCR reaction was carried out over 40 cycles with 15 seconds at 92°C, 30 seconds at 55°C and 20 seconds at 72°C in a volume of 50 µl.

Finally, scFv gene products were amplified by 1:25 dilution of the shuffling PCR reaction mixtures into a fresh 50 µl PCR reaction mixture, containing the primers Sfi1 and Sfi2 (Figure 6), and performing 30 cycles with 15 seconds at 92°C and 40 seconds at 72°C.

Phage panning

Library construction

*Sfi*I-digested and purified scFv genes resulting from DNA shuffling experiments were ligated into *Sfi*I-digested vector pAK100 (Krebber *et al.*, 1997), using 100 ng of vector DNA, 20 ng of insert DNA, 2.5 µl of 10× ligation buffer (NEB) and 600 units of phage T4 DNA ligase (NEB) in a volume of 25 µl. Mixtures were incubated at 16 °C overnight. *E. coli* XL1-blue was transformed with the ligation mixtures, using competent cells prepared according to Inoue *et al.* (1990), or by electroporation. Prior to electroporation, DNA was precipitated from ligation mixtures with *n*-butanol, and redissolved in 10 µl of water. Transformed cells were plated on 22 cm × 22 cm LB-agar plates (40 µg/ml chloramphenicol, 0.5% (w/v) glucose) and incubated at 37°C overnight.

Phage preparation

Colonies grown after overnight incubation were collected from plates and suspended in 10 ml of LB medium. Using 100 µl aliquots of these cell suspensions, infection with VCS helper phage, phage propagation and purification were carried out following standard protocols. The incubation temperatures for phage propagation were between 24°C and 30°C, depending on the selection pressure that was to be applied (see Results and Discussion).

Phage selection

Immunotubes (Nunc, Maxisorb) were coated overnight with antigen (bacterial levan from *Erwinia herbicola* (Sigma), 20 µg/ml in TBS (25 mM Tris-HCl (pH 7.5), 150 mM NaCl)) and blocked for 60 minutes at room temperature with 4% milk/TBST (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween-20). Panning tubes were incubated with 1 ml of phage solution (about 10^{12} colony-forming units in 2% milk/TBST) for 60 minutes at room temperature. For more stringent selection, 0.5 M GdnHCl was added during the binding incubation. After washing with TBST and TBS, bound phages were eluted using 1 ml of 0.1 M glycine-HCl (pH 2.2), by ten minutes incubation at room temperature. Eluates were immediately neutralized by addition of 60 µl of 2 M Tris base.

Reinfection

Usually, 1 ml of a freshly grown *E. coli* XL1 blue culture ($A_{550} = 0.5$) was infected with 250 µl of panning eluate, plated on LB-agar (40 µg/ml chloramphenicol, 0.5% (w/v) glucose) and incubated overnight at 37 °C. Resulting colonies were collected in 3 ml of LB medium, and phage propagation for the next panning round was carried out as described above.

Protein expression and purification

ScFv genes selected during phage panning experiments were recloned into vector pTFT74 (Freund *et al.*, 1993), which places expression under control of the phage T7 promoter, using standard cloning methods. ScFv proteins were expressed cytoplasmically, using strain *E. coli* BL21DE3 (F^-, *ompT*$^-$, $r_B^- m_B^-$, (λimm21, *lacI*, *lac*UV5, T7 pol, *int*); Studier & Moffatt, 1986), resulting in inclusion body formation. Purification of inclusion body protein, refolding and antigen affinity chromatography were carried out as described (Proba *et al.*, 1997), except that redox shuffling reagents were not applied during the refolding procedure.

Urea equilibrium d naturation

ScFv protein/denaturant-mixtures (2 ml) containing a final protein concentration of 5 µg/ml and denaturant concentrations varying from 0 to 6 M urea were prepared from affinity purified scFv protein solution and urea stock solution (9 M) in BBS (50 mM borate (pH 8), 150 mM NaCl). Exact urea concentrations in the final mixtures were determined by measuring the refractive indices. After overnight incubation at 10°C, the fluorescence emission spectra of the samples were recorded from 320 to 370 nm, at an excitation wavelength of 280 nm. A 3 ml cuvette was used, which allowed stirring of the sample during measurement, and the temperature was kept constant at 20°C. For every sample, five spectra were recorded and averaged. With increasing denaturant concentrations, the maxima of the recorded emission spectra shifted from about 336 to 348 nm. The maxima of the averaged spectra were determined by fitting a Gaussian function. The fraction of unfolded scFv protein, depending on denaturant concentration, was calculated according to Pace (1990).

Small-scale expression

SB medium (2 ml of 20 g/l tryptone, 10 g/l yeast extract, 10 g/l NaCl, 50 mM K_2HPO_4 containing 100 µg/ml ampicillin and 0.5 % glucose) was inoculated with a single bacterial colony and incubated overnight at 37°C: 25 ml of SB medium containing 100 µg/ml ampicillin (in a 100 ml flask) was inoculated with 0.5 ml of the overnight preculture and incubated at 30 °C with shaking at 200 rpm. Expression was induced by addition of IPTG to a final concentration of 1 µM at $A_{550} = 0.5$ and incubation was continued for another three to four hours. Cultures were centrifuged (5000 *g*, ten minutes, 4°C), and cell pellets suspended in PBSE (25 mM phosphate buffer (pH 6), 150 mM NaCl, 1 mM EDTA), containing 5 mM bis-2-mercaptoethylsulfone (BMS), or in TBSE (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA) in the presence or absence of 10 mM DTT, normalizing the cell densities of the samples to $A_{550} = 25$. Cell disruption was achieved by French Press lysis, the resulting crude extracts were centrifuged (15,000 *g*, ten minutes, 4°C) and the supernatants used for ELISA and Western blot analysis.

Western blots

Samples of soluble fractions of crude extracts (see above) were subjected to SDS-PAGE, in the presence or absence of β-mercaptoethanol, and gels were blotted onto PVDF membranes. For immunodetection, the monoclonal anti-myc antibody 9E10 (Munro & Pelham, 1986) (1:5000 in TBST, 60 minutes at room temperature) was used, followed by incubation with a polyclonal anti-mouse-peroxidase conjugate (Pierce) (1:5000 in TBST, 60 minutes at room temperature).

ELISA

ELISA plates (Nunc) were coated with a solution of levan from *E. herbicola* (Sigma: 10 µg/ml in TBS overnight at 4°C) and blocked for 60 minutes at room temperature, using a solution of 4% milk in the same buffer used for sample preparation. Samples of the soluble fractions were premixed with different amounts of bacterial levan (0.025% and 0.25% (w/v), 100 µl volume) and incubated on levan-coated ELISA plates for 60 minutes at room temperature. Bound scFv protein was detected using the monoclonal anti-myc-tag antibody 9E10 and a polyclonal anti-mouse/peroxidase conjugate (Pierce).

Phage-ELISA

Phage suspension (50 µl corresponding to 4 ml of phage culture precipitated and resuspended in TBST) was preincubated with 50 µl of 2% skimmed milk/TBST in the presence or absence of 0.025% levan for ten minutes, then applied to levan-coated, milk-blocked ELISA plates for one hour at room temperature. Bound phages were detected using an anti-M13 antibody/peroxidase conjugate (Pharmacia). The phage titers were determined by reinfection, and the ELISA signals were normalized to a constant phage number.

Molecular modelling

The structures of wild-type, disulfide-free and disulfide-restored A48 Fv fragments were modelled based on the X-ray structures of the V_H domain of the anti-phenylarsonate antibody Fab 36-71 (PDB file 6fab, resolution 1.9 Å, 87.7% sequence identity (Strong *et al.*, 1991)) and the V_L domain of anti-galactan antibody J539 (PDB file 2fbj, resolution 1.95 Å, 85.6% sequence identity (Bhat *et al.*, unpublished)) as described previously (Proba *et al.*, 1997).

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